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Amphotericin-B-Loaded Polymersomes Formulation (PAMBO) Based on $(PEG)_3$-PLA Copolymers: An in Vivo Evaluation in a Murine Model

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Abstract: This paper deals with in vivo evaluation of a new amphotericin-B-loaded polymersomes (PAMBO) formulation in terms of pharmacokinetics, toxicity, tissue distribution profile, and its efficacy in a murine model of disseminated candidiasis. Pharmacokinetic and tissue distribution studies of the PAMBO showed sustained levels of the drug in plasma as well as in target organs which harbor fungal and leishmanial infection. PAMBO was found to be much less toxic than Fungizone. It was observed that 700% increment in the dose is tolerated without observable toxicity which is supported by survival, biochemical, and histopathological results. PAMBO showed a significant improvement in the survival rate of immunosuppressed mice infected with *Candida albicans* as compared to control. It also showed better dose to dose (1 mg/kg) efficacy as compared to Fungizone and a significant improvement in the life expectancy at 3 and 5 mg/kg dose levels in the animals. Colony forming unit (CFU) counts in the target organs revealed significant reduction in *Candida* burden with PAMBO treatment. Kidney, spleen, and lung were cleared of infection, although liver was carrying a very low level of infection. Overall, PAMBO formulation is found to be more efficacious and less toxic in a fungal mice model.

Keywords: Nanomedicine; amphotericin B; polymersomes; toxicity; candidiasis

Introduction

Amphotericin B (AmB) is a broad spectrum antifungal and anti-leishmanial drug; however, it is a Biopharmaceutics Classification System (BCS) class IV drug and is difficult to formulate for oral administration to produce systemic effects.[1] Hence, it has been formulated conventionally for i.v. injection in the form of micelles of bile salt (sodium deoxycholate) or using polymers and marketed as Fungizone for the past six decades.[2−5] Though Fungizone is a marketed formulation, however, its delivery is complicated because

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of frequent adverse effects that include fever, chills, nausea, vomiting, and hypokalaemia in addition to severe neprhotoxicity.[6−8] Fungizone is unable to hold the drug within the micelle, and after i.v. injection the free drug causes associated adverse effects. Nephrotoxicity occurs mainly because of interaction of AmB with cholesterol which is abundant in renal tubular cells. Prolonged administration of AmB results in elevated serum creatinine and urea levels. Serum creatinine levels indicate the degree of toxicity and hence influence the treatment regimen.[9,10] This nephrotoxicity usually becomes more pronounced as therapy continues and results in irreversible loss of kidney function.

Three novel lipid-containing formulations of AmB have been developed in an attempt to attenuate its nephrotoxicity and increase its therapeutic potential. These formulations include, but are not limited to, AmB lipid complex (ABCL or Abelcet), AmB colloidal dispersion (ABCD or Amphotec), and small unilamellar vesicle formulations of AmB (Ambisome). However, their higher economic costs and instability because of the phospholipids are a major limitation in clinical practice. Moreover, it has been recognized that the reduction in AmB toxicity was associated with a substantial reduction in AmB activity. Several infrequent side effects have been reported in patients, including allergic reactions,[11] cardiopulmonary toxicity,[12,13] and severe systemic side effects.[14]

To curb the phospholipids from the formulation, various polymer formulations of AmB have been developed which include micro- and nanoparticles.[15,16] However, until date no vesicular type of polymeric formulation has been reported for AmB. We hypothesized that the vesicular formulation (polymersomes) are made up of amphiphilic block copolymers and hence can behave similar to amphiphilic phospholipids in entrapping and interacting with AmB. At the same time, this polymersomes formulation of AmB would be of less cost and of higher stability.

Keeping this hypothesis, we have synthesized an amphiphilic block copolymer having three poly(ethylene glycol) (PEG) chains and a long poly(lactic acid) (PLA) chain attached via a citric acid linker. The polymer was found suitable for forming polymersomes.[17] AmB loaded polymersomes (PAMBO) formulation was developed and found to be much less toxic than Fungizone when evaluated in vitro.[18] The prepared formulation was further stabilized by lyophilization. In this paper, authors report the in vivo assessment of the prepared formulation with respect to pharmacokinetics, tissue distribution, and toxicity, which is a major concern for AmB and efficacy in disseminated fungal model.

Table 1. Chacteristics of the Formulation (PAMBO) Evaluated in in Vivo Experiments

polymer	size of polymersomes	zeta potential	loading
$(PEG_{1100})_3$-PLA 1:9 (PGCL19)	266.45 ± 34.47 nm	−16.34 ± 2.54 mV	16.26 ± 2.50%

Experimental Section

Amphiphilic block copolymer (PGCL19) composed of dl-PLA and mPEG was synthesized in-house as reported earlier.[17] AmB was a generous gift as free samples from Ambalal Sarabhai Chemicals (Pvt.) Ltd. (Vadodara, India). Amphotericin B loaded polymersomes (PAMBO) were prepared in our lab as described in our previous report,[18] and details of the formulation used in this study are given in Table 1. Fungizone was purchased from a local market, manufactured by Ambalal Sarabhai Enterprises Ltd. (Vadodara, India). The internal standard 1-amino-4-nitro naphthalene was procured from Sigma (USA). Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA \cdot 2H_2O$) AR grade was purchased from Loba Chemie

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(Mumbai, India), and HPLC grade acetonitrile was obtained from J. T. Baker (USA). Emmons modified Sabouraud dextrose agar media (Emmons modified SDA) was purchased from Himedia (Mumbai, India). Ultrapure water prepared from a water system (Elga Ltd., Bucks, England) was used in the study.

Animals. Male SD rats (275−325 g) were used for pharmacokinetic study, while male Balb/C mice (15−20 g) were utilized for tissue distribution and toxicity of the formulations. Female Balb/C mice were used for efficacy studies. Animals were housed (rats in groups of three and mice in groups of four) in plastic cages in a 12 h dark−light cycle, with controlled temperature (25 °C) and humidity (70%). Water and food were provided ad libitum throughout the study. The animals were housed in Central Animal Facility (CAF) of National Institute of Pharmaceutical Education and Research (NIPER). All protocols were approved by Institutional Animal Ethics Committee (IAEC), and experiments were performed in accordance with CPCSEA.

Pharmacokinetic Study of AmB Formulations. Ten male SD rats were randomly divided in two groups of five each, and each animal has received a single formulation having 1.0 mg/kg AmB equivalent dose. Such groups were made for all AmB formulations, namely, Fungizone, Ambisome, and PAMBO. A single intravenous bolus dose was injected via the femoral vein in a total volume of 1 mL/kg, based on each animal's body weight measured on the day prior to dosing. Plasma samples were obtained at 5 and 30 min, and 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, 96, and 120 h after dosing. In this experiment, samples were taken alternatively from each group so that the blood loss could be maintained at minimum in one animal at the time of injection. This was done because of the fact that AmB is highly bound to plasma lipoproteins, and thus, this technique helped to minimize the potential effects of blood loss on the observed kinetics. Plasma was separated by centrifugation from blood samples of volume size 0.5−1.0 mL and stored frozen at temperature less than −20 °C. For HPLC analysis, the samples were processed in the following manner: samples were thawed at RT, and 50 μL of internal standard solution (1 μg/mL solution of 1-amino-4-nitronaphthalene (ANN)) was added, mixed followed by addition of 1.4 mL of methanol, and vortexed. The sample mixture was centrifuged at 18 000*g* for 10 min, and the supernatant was collected in another tube. The supernatant was dried in a centrivac (Maxi dry lyo, Denmark) and reconstituted in 120 μL of mobile phase. Recovery of AmB from spiked plasma samples was ∼90% with the assay sensitivity at 20 ng/mL in plasma.

Pharmacokinetic Data Analyses. Data were analyzed by Kinetica software (Thermo Fischer Scientific, USA) using noncompartmental modeling. The last three points of mean values of the plasma concentration versus time plot were used to calculate the "area under the curve" extrapolated to infinity and to estimate terminal half-lives ($t_{1/2}$).

Tissue Distribution and Toxicity Study. The study was carried out at different dose levels of Fungizone and PAMBO formulation. Sixteen male Balb/C mice weighing 15−20 g were randomly divided into four groups of four animals each for every dose level and type of formulation. Doses for Fungizone and PAMBO were 1, 1.5, 3, and 5 mg/kg and 1, 3, and 6 mg/kg AmB equivalent, respectively. Each animal received a single dose of single formulation as i.v. bolus injection via the tail vein with a total volume of 100−200 μL. The volume of injection was dependent on the body weight of each animal prior to dosing. Animals were sacrificed at four time points, namely, 5 min, 4 h, 24 h, and 96 h after taking blood samples. Liver, kidneys, spleen, and lungs were excised, washed thoroughly with phosphate-buffered saline (PBS), and homogenized in PBS, and 200 μL aliquots were stored at −60 °C (Ultralow freezer, Vestfrost, Denmark) before analysis. The volume of PBS for homogenization was kept at 1:3 (w/v) of tissue in all cases except spleen, where it was kept at 1:5 (w/v) because of less weight of the organ. For analysis, samples were thawed at RT and 50 μL of internal standard solution (1 μg/mL solution of ANN) was added, mixed followed by the addition of 700 μL of methanol, and vortexed. The sample mixture was centrifuged at 18000*g* for 7 min, and the supernatant was collected in another tube. The pellet was resuspended in another 700 μL of methanol with the help of sonication and centrifuged again at 18 000*g* for 10 min. The supernatant was withdrawn and pooled to the earlier fraction. Extraction was carried out twice to extract the maximum amount of the drug. The recovery of AmB from spiked tissue samples was between 70 and 110%. The assay sensitivity was ≤50 ng/g of the tissue.

For toxicity evaluation of formulation, two parameters, namely, mortality and nephrotoxicity, were studied. For the mortality study, the animals were given different doses of formulations and were kept under observation for 1 h; animals which survived were used for nephrotoxicity and tissue distribution studies. Nephrotoxicity was evaluated by using two renal function tests, namely, plasma creatinine (PCRT) and blood urea nitrogen (BUN), and by histopathological studies of kidney. For this purpose, heparinized blood samples were used and plasma was obtained after centrifugation. The biochemical measurement was carried out by using ready-made kits (Accurex Biomedical Pvt. Ltd., Mumbai, India) using the manufacturer's protocol. Absorbance of the samples was measured by using a spectrophotometer plate reader (PowerWave XS2, BioTek Instruments, USA) in conjunction with Gen 5 software.

For histopathology, excised kidneys were fixed in 10% formal saline for at least 48 h. The tissue was processed for routine histopathology. All the samples were dehydrated by placing them in an increasing gradient of absolute alcohol and xylene. The anhydrous tissue samples were then embedded in paraffin blocks. Five micrometer thick ections of the tissue were cut with the help of a microtome and processed for hydration and staining by hematoxylin and eosin dyes. Renal morphologic characteristics were evaluated under light microscope for assessing tubular and glomeruli damage in the sections by a proficient pathologist.

Efficacy Studies. For this purpose, a murine disseminated candidiasis model was developed and optimized for the evaluation of antifungal activity of the developed formulation.

Candida albicans and Media. Candida albicans (*C. albicans*) obtained from the American Type Culture Collection (ATCC) 90028 (blood isolate from Lowa, submitted by MA Pfaller) and maintained at the National Culture Collection for Pathogenic Fungi (NCCPF) in the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, was used in this study. Further, the strain was brought to NIPER from PGMIR in tube-culture and was subcultured. The strain was maintained and stored as glycerol stocks (10% glycerol in ultrapure water) at −60 °C until further use. For subculture, Emmons modification of Sabouraud dextrose agar media (SDA) was used.

Preparation of Inoculum. Cultures were revived from glycerol stock onto SDA slants for 48 h at 35 °C before use. Cells were suspended by vortexing a single pure colony in pyrogen free normal saline and subsequently diluted to obtain a cell population between 1.25×10^6 and 2×10^6 cells/mL. Cells were counted using a hemocytometer and then suitably diluted to obtain the required number of conidia per milliliter for infection. The colonies were pure as identified from the morphology, and none of the cell suspensions were contaminated with any other organism.

Efficacy in Fungal Model. Optimization of the fungal model was carried out in terms of the dose regimen of cyclophosphamide for immunosuppression and the number of *Candida* cells injected per animal for infection. Balb/c mice were rendered immunocompromised by injecting a single dose of cyclophosphamide (150 mg/kg) 48 h before infection. Systemic (disseminated) candidiasis was developed by injecting 1×10^4 *C. albicans* cells in 125 μL of sterile normal saline per mouse via the tail vein. Six hours later, animals were administered either as a single i.v. bolus injection Fungizone (1 mg/kg, $n = 8$), PAMBO (1, 3, 5 mg/kg, $n = 10$), or physiologic saline (untreated controls; $n = 6$). Animals were observed for 14 days postinfection for survival. On the 15th day, two animals from each group were sacrificed out of the surviving animals. From the sacrificed animals, organs (liver, kidney, spleen, and lung) were excised and aseptically homogenized in 5 mL of pyrogen free sterile saline. The homogenate was diluted suitably with saline for plating on SDA media. Fifty microliters of tissue suspension was spread on the plate, which was sealed with parafilm and incubated at 35 °C for 48 h before colony counting. Colony forming units (CFUs) were counted using a colony counter (BZG30, Carl Stuart Limited, UK). In control animals, this experiment was performed after 24 h of infection.

Results

Pharmacokinetic Study. In this study, mean plasma profile of AmB formulations was found to follow two compartment models from the semilog plot. The plasma profiles of all three formulations are given in Figure 1, and

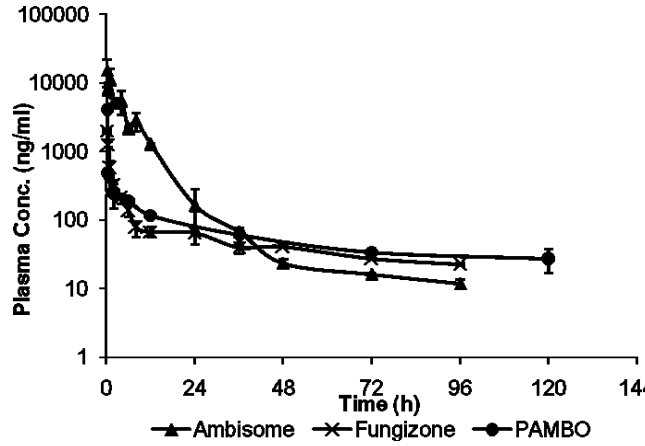

Figure 1. Semilog plasma profiles of AmB formulations ($n = 5$).

Table 2. Pharmacokinetic Parameters of AmB Formulations

formulation (1 mg/kg AmB equiv)	$C_{5\ min}$ (ng/mL)	$T_{1/2}$ (h)	$AUC_{0-\infty}$ (ng/mL·h)	CL (mL/h)	Vd (mL)
Fungizone	1998.68	64.83	8511.82	117.48	10 990.57
Ambisome	15 372.60	27.31	62 218.76	16.072	633.38
PAMBO	3997.83	73.84	11354.29	88.072	9384.23

the pharmacokinetic parameters are shown in Table 2. It can be seen that there are pronounced differences in plasma concentration−time profiles and the calculated pharmacokinetic parameters derived from i.v. bolus administration of commercial formulations and the developed formulation PAMBO. It is observed that the pharmacokinetics of AmB was highly dependent on the carrier system. It can be seen from Figure 1 that the maximum plasma concentration of AmB was observed at 5 min which is also the first sampling point; hence, this time is treated as C_{max} for all the formulations. The PAMBO formulation showed C_{max} (3997.83 ng/mL) less than that of Ambisome (15 372.6 ng/mL) but more than that of Fungizone (1998.68 ng/mL). $T_{1/2}$ of PAMBO was higher than that of the other two formulations. The area under the curve ($AUC_{0-\infty}$) for PAMBO formulation was 11 345.29 ng/mL·h which was higher than that of Fungizone and lesser than that of Ambisome (Table 2). Drug clearance from the blood compartment and the volume of distribution follow the trend Fungizone > PAMBO > Ambisome.

Toxicity Study. Toxicity of the formulation (PAMBO and Fungizone) was evaluated by the mortality of animals at different dose levels and biochemical parameters (PCRT and BUN) at different time points. Figure 2 shows the mortality in the animals within 1 h of injection. All animals died in the case of Fungizone at dose levels of ≥3 mg/kg, whereas no death was observed with the PAMBO formulation at dose levels up to 6 mg/kg. In case of Fungizone, one animal in a group of eight died at the dose of 1 mg/kg and two animals died at 1.5 mg/kg. The results are in concordance with the literature for Fungizone, where the maximum tolerated dose

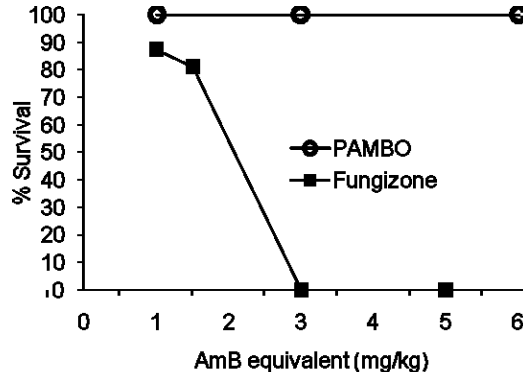

Figure 2. Survival of Balb/C mice after administration of a single i.v. bolus injection at different dose levels of AmB formulations.

(MTD) is reported to be 0.8−2 mg/kg in the case of Balb/C mice.[19−21] Only five animals were injected with a higher dose, as all of them died within 0.5 h of the injection.

Figure 3A and B shows the PCRT and BUN levels, respectively, at different time points after injection of a single i.v. bolus of AmB formulations. For Fungizone, PCRT levels, at the dose of 1 mg/kg, rose to more than 200% of the control and maintained almost the same levels even after 96 h. When the dose was increased to 1.5 mg/kg, the levels increased by more than 300% and reached a maximum of 376% after 24 h. The levels of changes in the PCRT were found to be statistically significant ($P < 0.005$). PAMBO did not show any significant change in the PCRT levels in comparison to the control up to a dose of 6 mg/kg. During renal toxicity testing with AmB, glomerular and renal tubular damage in kidney tissues occurs,[22] which can be observed in the histopathology of kidney sections of treated animals. The histopathology of a kidney section of a treated animal using Fungizone and PAMBO at different dose levels is shown in Figure 4.

Tissue Distribution Study. For this purpose, tissue distribution of AmB for both Fungizone and PAMBO at 1 mg/kg dose and PAMBO at 3 mg/kg was assessed in four target tissues, namely, liver, spleen, kidney, and lungs, and profiles are shown in Figure 5. It is observed that, at the same dose level for both formulations, the AmB concentration is lower for PAMBO in comparison to Fungizone; however, in the later phases, PAMBO was able to maintain

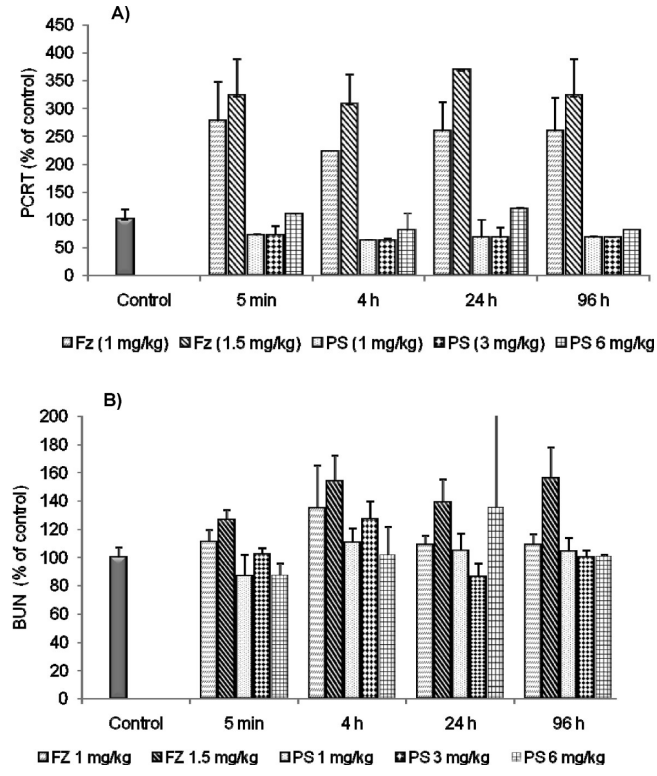

Figure 3. (A) PCRT and B) (BUN levels of Balb/C mice at different time points after i.v. bolus injection of AmB formulations. The values are represented as percentage of control. Fz and PS are Fungizone and PAMBO formulations, respectively ($n = 4$).

the concentration of AmB in the target tissues. At the same time, the drug levels in targeted tissues were exceptionally higher at 3 mg/kg equivalent AmB dose level of PAMBO; however, this evaluation could not be done in the case of Fungizone, as no animal survived at this dose level.

Efficacy Studies. The efficacy of the formulation was assessed on the basis of two parameters, namely, survival and CFU count in target organs. For survival study, Kaplan−Meier statistical analysis was performed in order to see if AmB formulations prolonged the survival as compared to control. Figure 6 shows the Kaplan−Meier plot of survival in different treatment groups along with control group. As a part of Kaplan−Meier analysis, the comparison of survival curves was performed by means of function survfit, and the results of the log-rank test for the difference between both groups are shown in Table 3. The results obtained were statistically significant between control and all other treatment groups. It can be seen from the analysis that all the treated groups had better survival than the untreated control group of mice infected with *C. albicans*. All the animals in the control group died within 48 h as indicated by a 0.00 survival rate on day 2. The Fungizone treated group at 1 mg/kg had a survival rate of 0.71 and 0.00 on days 2 and 7, respectively. PAMBO on the other hand showed 0.71, 1.00, and 1.00 survival rates at day 7 for 1, 3, and 5 mg/kg dose, respectively. High χ^2 values and very low P values indicate the high level of statistical

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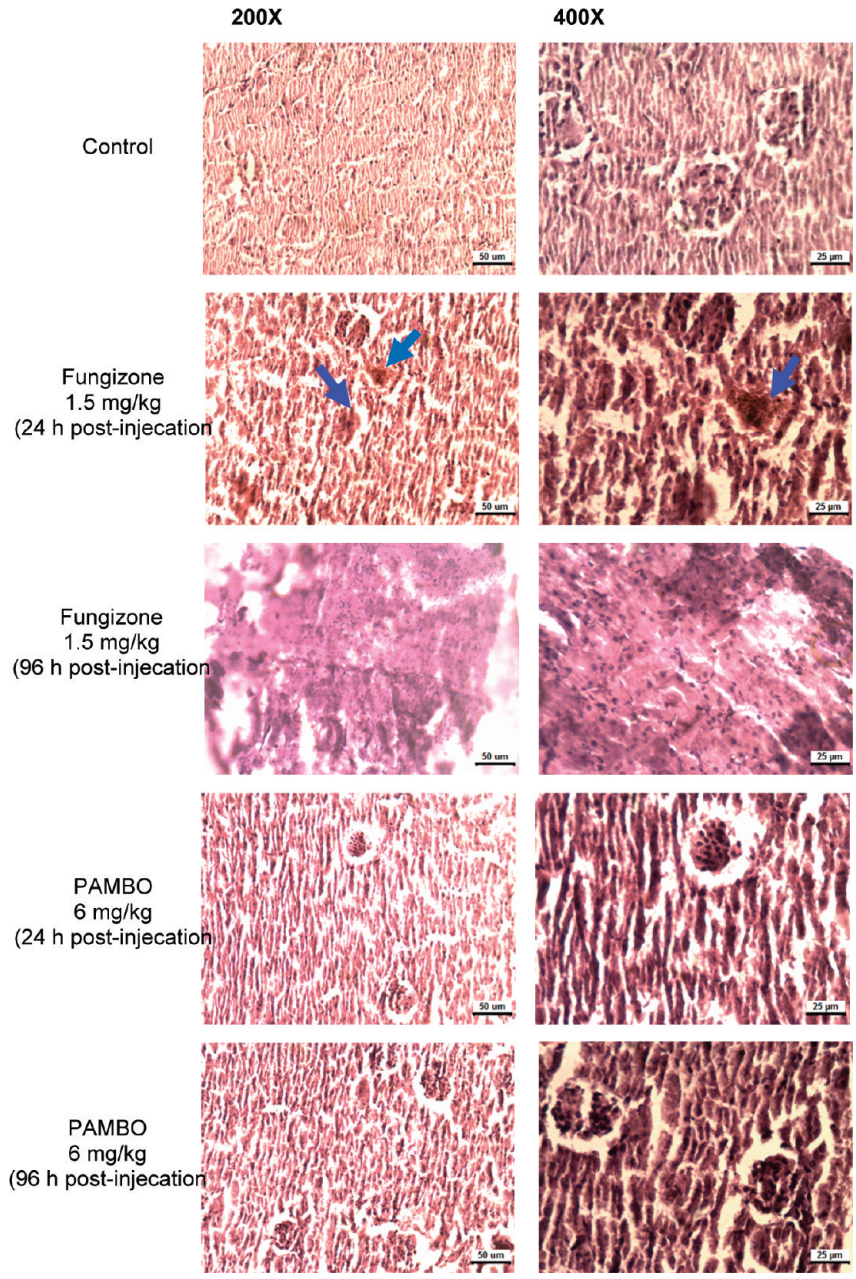

Figure 4. Photomicrographs of kidney sections of Balb/C mice after a single dose administration of Fungizone and PAMBO for histopathological evaluation. No major histopathological changes were observed at any dose level because of single dose study except for neutrophil infiltration, inflammation (marked with arrows) after 24 h, and necrosis after 96 h with Fungizone at 1.5 mg/kg dose.

significance in data analysis. PAMBO at 3 and 5 mg/kg dose had highly significant improvement in the life expectancy for treated groups, as can be seen from the high χ^2 values and very low P values obtained for the comparison of control animals. Sustained release of AmB from PAMBO and thus continuous exposure of *C. albicans* to AmB could be the reason for improved survival as compared to Fungizone even at a similar dose level of 1 mg/kg. However, this was not significant. At the same time, PAMBO at 3 and 5 mg/kg dose showed a significant improvement in the survival ($P < 0.0001$) of mice in comparison to Fungizone. Overall, PAMBO showed a good improvement in the survival of *C. albicans* infected mice in comparison to control as well as Fungizone at the same and high dose levels. *C. albicans* organ burden for the liver, kidney, spleen, and lung was analyzed in surviving animals on day 15 postinfection, and results are shown in Figure 7. In control animals, the count was made after sacrificing animals 24 h postinfection.

Discussion

Pharmacokinetics studies of all three formulations gives insight into the fate of a drug molecule in the blood compartments; however, it may be different for nanoformulations in comparison with simple drug molecules. Specifically, nanoformulations may release the drug molecules in the blood compartment or may carry them directly to the

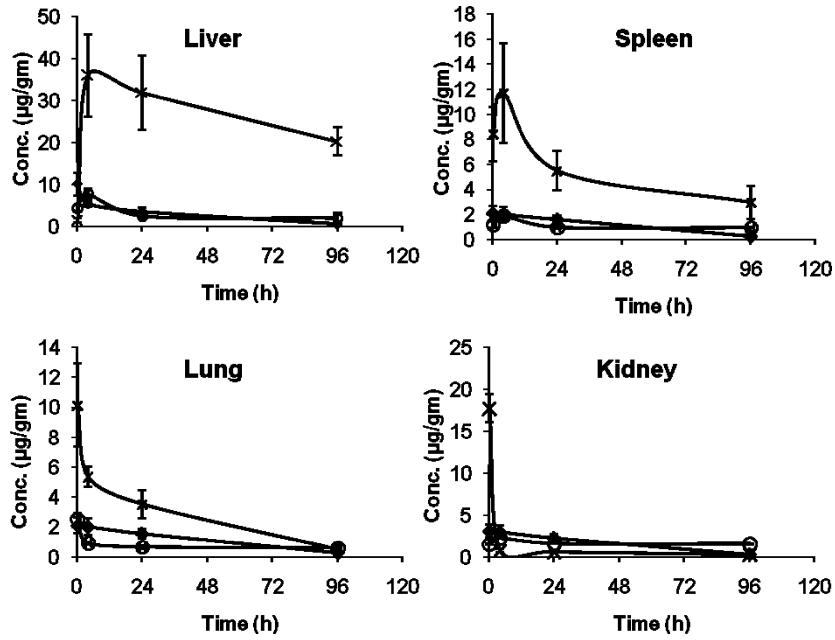

Figure 5. AmB concentrations at various time points in the liver, kidneys, spleen, and lung of Balb/C mice after a single i.v. dose of PAMBO and Fungizone. Each point shows the mean ± standard deviation for four animals. Symbols: (◆) Fungizone (1 mg/kg), (○) PAMBO (1 mg/kg), and (×) 3 mg/kg (3 mg/kg).

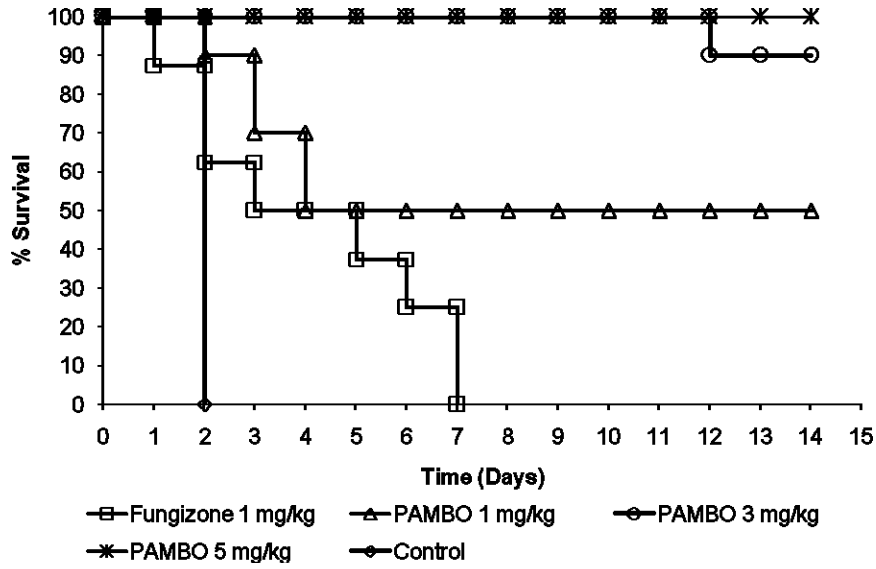

Figure 6. Kaplan−Meier survival plot of Balb/C mice infected with *C. albicans* and treated with Fungizone at 1 mg/kg and PAMBO at 1, 3, and 5 mg/kg dose levels along with untreated (control) group. $n = 6$ for control group, $n = 8$ for Fungizone, and $n = 10$ for all groups of PAMBO.

Table 3. Analysis of Survival Data in Fungal Model by Kaplan−Meier Method

treatment (dose in mg/kg)	n[a]	mean survival time	survival rate at day 2/day 7	O[b]	E[c] (control/treatment)	P value (χ^2)
control	6	1.70 (0.02)	0.00/0.00	6	NA[d]	NA[d]
Fungizone (1)	8	4.13 (0.11)	0.71/0.00	8	2.6/11.4	<0.05 (5.46)
PAMBO (1)	10	O < n	0.90/0.71	5	1.5/9.5	<0.001 (15.95)
PAMBO (3)	10	O < n	1.00/1.00	0	1.25/5.75	$<1 \times 10^{-5}$ (21.97)
PAMBO (5)	10	O < n	1.00/1.00	0	1.25/4.75	$<1 \times 10^{-5}$ (22.80)

[a] Sample size. [b] Observed no. of deaths. [c] Expected number of deaths. [d] NA: Not applicable.

tissue compartment. Thus, pharmacokinetics of drug molecules in carrier systems or as free molecules may behave differently and is highly dependent on the type of carrier system. It is also important to note that in vitro drug release from PAMBO is slower and the size of PAMBO is higher than that of the other two formulations. After extrapolating

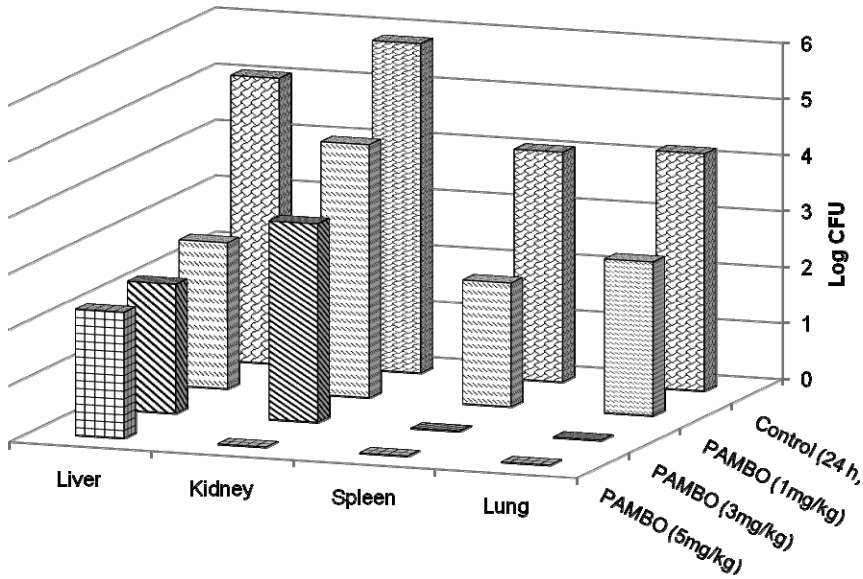

Figure 7. *C. albicans* burden in various organs of Balb/C mice of control (24 h postinfection) and PAMBO treated groups (15 days postinfection). CFU counts were significantly low at all dose levels in all the studied organs. Kidney, spleen, and lung were cleared from the infecation at AmB dose of 5 mg/kg of PMABO formaution. The low level of infection in the liver may not be *Candida*, as the morphology of the colony was dissimilar to that of the candia colony.

these results in vivo, It is expected that the drug release may also be faster in the case of Ambisome which results in a maximum drug concentration in the blood compartment as well as higher C_{max}, and the same was observed in the in vivo results (Table 2 and Figure 1). A sharp decrease of drug concentration in plasma was also observed in the case of Ambisome, and this may be due to exhaustive release from the liposomal carrier system. PAMBO, on the other hand, maintained higher plasma concentration than other two formulations after 36 h and moreover maintained it for longer time duration. Slow release of the drug from polymersomes supports the higher $T_{1/2}$ and lower C_{max} of PAMBO during pharmacokinetics studies. The lower AUC of PAMBO may be due to the fact that drug remained in the carrier system and drug might have reached the tissue compartment directly. This supported by the other section of tissue distribution study as drug concentration in targeted tissues was higher in PAMBO in comparison to Fungizone. This particular phenomenon may be clinically beneficial for treatment of fungal as well as leishmanial infection as drug concentration is maintained for longer duration of time in the body.

The toxicity of the formulations (PAMBO and fungizone) was evaluated at different dose levels to the maximum tolerable dose in the animal during treatment. For this purpose, four doses of fungizone (1, 1.5, 3, and 5 mg/kg equivalent of AmB) and three doses of PAMBO (1, 3, and 6 mg/kg equivalent of AmB) were given to the animals, and it was observed that all animals at the dose higher than 1.5 mg/kg equivalent of AmB of Fungizone died; however, 90 and 80% survival was seen for the dose level of 1 and 1.5 mg/kg equivalent of AmB of Fungizone. On the contrary, all animals survived with all the test dose levels of PAMBO formulation. This might be because of slow drug release and low exposure of AmB molecules in the case of PAMBO which also indicates that there is no toxicity of PAMBO formulation at all the tested dose levels.

The toxicity of any formulation can also be measured by the evaluation of PCRT and BUN levels, wherein higher levels of PCRT and BUN indicate higher degree of toxicity. The mechanism for increase in the PCRT is due to renal parenchymal damage which decreases creatinine filtration and, in turn, increases PCRT levels and indicates the renal toxicity. In this experiment, PAMBO formulation did not show any increase of PCRT levels at all tested dose levels which indicates almost no renal toxicity up to a dose level of 6 mg/kg, as can be observed (Figure 3A). It can be seen in Figure 3B that there is no significant change in BUN levels for all formulations after single dose administration, although BUN levels were always slightly higher in the case of Fungizone. In the case of PAMBO, higher BUN levels were observed in only one animal 24 h after injection at 6 mg/kg dose. The renal toxicity is also evaluated by increased BUN level; however, the mechanism for the change in BUN is different compared to PCRT as it is affected by two balancing parameters which increase BUN levels due to reduced filtration for the same reasons as stated for creatinine. At the same time, BUN is decreased by reduction in its reabsorption because of damage to renal proximal tubules. Overall, it may result in insignificant changes in BUN levels in single dose studies, and the same was observed in the case of PAMBO formulation at all tested dose levels.

In the present study, histopathology results did not revealed any major histopathological changes at the studied dose levels except for neutrophil infiltration and inflammation (marked with arrows) after 24 h and necrosis after 96 h with Fungizone at 1.5 mg/kg dose (Figure 4). Overall, PAMBO shows lower nephrotoxicity in comparison to Fungizone. This study is a single dose study which indicates that the

maximum dose which can be injected safely in the case of PAMBO could be as high as ~700% in comparison to conventional Fungizone formulation.

The fate of any drug formulation is dependent on its distribution profile in various compartments of the body. Distribution of formulation in various organs imparts efficacy or may create the toxicity to particular organ(s). Thus, along with the pharmacokinetic studies, it is also important to see the distribution levels of the drug in various organs and optimize the formulation for its better efficacy and lower toxicity. For tissue distribution studies, two dose levels (1 and 3 mg/kg) were selected for optimized animal usage with full outcome of the studies. On the evaluation of drug levels in liver, spleen, lung, and kidney after the dose of 1 mg/kg equivalent AmB of Fungizone and PAMBO, it was observed that, at early time points, drug levels were slightly lower in the case of PAMBO formulation in comparison to Fungizone; however, these level were higher at the latter time point (96 h). It is also a fact that drug release from PAMBO formulation is slower due to controlled release properties of these carriers. This was also seen during in vitro drug release studies; however, due to continuous release of the drug, these levels were higher at the 96 h time point which is a positive outcome of this study for PAMBO formulation. The results at higher dose levels, that is, at 3 mg/kg equivalent AmB are also supportive of this, as drug levels were exceptionally higher in target tissues (Figure 5). This indicates that sustained release of AmB occurred in the case of PAMBO which may result in longer duration of action and may be therapeutically beneficial in treating fungal and leishmanial infections. On increasing the dose of PAMBO to 3 mg/kg, a nonlinear kinetics and disproportionate increase in the AmB tissue concentration was observed which is quite obvious as distribution of drug is due to (1) free drug released in the blood compartment and (2) transportation of the drug entrapped in polymersomes directly to the tissues which is a normal process. Lower AUC in pharmacokinetics studies and higher drug levels in tissues also support this understanding. It is important to note that PAMBO formulation is nontoxic up to 6 mg/kg AmB equivalent and thus an increase in drug concentration in target organs at a higher dose up to 6 mg/kg can further be utilized for therapeutic efficacy and at the same time lower toxicity.

In efficacy studies, a clear relationship was observed between the AmB equivalent dose and *Candida* burden in the organs. The survival rate of the infected animal was better in the animals treated with PAMBO formulation at equivalent dose level in comparison to Fungizone (Figure 6). It was far better at higher dose levels of PAMBO, and it was 90% and 100% at 3 and 5 mg/kg equivalent AmB of PAMBO, respectively; however, it could not be seen in Fungizone at these dose levels as no animal could survive due to toxicity of the Fungizone. A similar trend was observed in all three targeted organs: liver, spleen, and lung tissues. However, the same trend could not observed in kidney tissues where drug levels were quite low in the case of PAMBO formulation at both dose levels. In corroboration with the literature reports, *Candida* was found to accumulate more particularly in kidney. Liver was another organ which was found to have a higher *Candida* load. Spleen and lung, on the other hand, were found to have low *Candida* burden and thus cleared from the infection completely even though the drug concentrations were low in comparison to other two organs. These two organs became sterile at 3 mg/kg AmB equivalent dose of PAMBO. *Candida* burden in the liver was reduced to a significantly low level ($P < 0.002$) at 1 mg/kg AmB equivalent, as the delivered AmB concentration was higher in liver than in other organs with PAMBO. The colonies found in the liver at 3 and 5 mg/kg dose had a different morphology which could be due to other opportunistic infection or lower levels of the drug in the organ. At 1 mg/kg dose, a significant reduction in CFUs was found in kidney ($P < 0.007$), lung ($P < 0.015$), and spleen ($P < 0.002$) in comparison to control. At a dose of 3 mg/kg AmB equivalent, a further reduction of *Candida* burden was found in the kidney as compared to the reduction at 1 mg/kg. At a dose level of 5 mg/kg, no *Candida* burden was observed in kidney. The CFU data reveals direct correlation of dose and survival of the animals infected. The efficacy study indicates that PAMBO is a better formulation for antifungal treatment in comparison to Fungizone.

Conclusions

A new polymersomes based formulation of amphotericin B (PAMBO) was developed and tested in vivo where pharmacokinetic studies suggest sustained levels of drug in the blood compartment and tissues of target organs while toxicity studies suggested that PAMBO is more tolerable up 6 mg/kg AmB equivalent and 700% of conventional Fungizone marketed formulation. Efficacy studies suggested the better survival of animals at an equivalent dose of PAMBO than Fungizone; however, complete survival up to 10 days of evaluation period with 5 mg/kg AmB equivalent of PAMBO in comparison to total death of animals at 3 mg/kg AmB equivalent of marketed formulation Fungizone suggested the higher efficacy of PAMBO in comparison to marketed formulation. In vivo results in animal models suggest that PMABO is safer then conventionally used Fungizone formulations and has high efficacy in a disseminated fungal animal model.

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Supporting Information Available: Chemical structures related to the formulations used in this paper. This material is available free of charge via the Internet at http://pubs.acs.org.

Note Added after ASAP Publication. The Experimental Section contained an error in the source of Fungizone in the version published ASAP on December 17, 2010; the corrected version was reposted on December 23, 2010.

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